Behaviour of the X chromosomes during growth and maturation of parthenogenetic eggs of *Amphorophora tuberculata* (Homoptera, Aphididae), in relation to sex determination

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Abstract. Prophase chromosomes of growing oocytes from thelytokous, viviparous females of *Amphorophora tuberculata* Brown and Blackman ($n = 2$) were studied using a modified propionic acid squash technique with Feulgen staining. In early prophase, prior to the growth phase of the oocyte, the X chromosomes are partially condensed and looped together so that all four ends appear to be associated. Later in prophase the X chromosomes separate in oocytes destined to be female, but remain associated in presumptive male oocytes. The autosomes condense gradually throughout prophase. The nucleus of the presumptive male oocyte is further characterised by the formation of a spherical Feulgen-positive body, which attains a large size (7 μm diameter) in late prophase. At this stage, the X chromosomes are no longer visible as separate entities, and are apparently included in the spherical body. At metaphase this disappears, leaving the X chromosomes still united as a condensed bivalent. The spherical body seems to have nucleolar as well as chromatin constituents; nucleolar organisers are present at the ends of the X chromosomes where it first arises. It may function in maintaining the cohesion between the X chromosomes through prophase, and could also facilitate correct orientation of the X bivalent on the spindle of the maturation division. As sex determination in aphids is controlled by juvenile hormone concentration, it appears that the hormone may interact with the X chromosomes during prophase, bringing about their separation in female oocytes, perhaps by inhibiting the formation of the spherical body.

Introduction

Despite the economic importance of aphids our understanding of their genetics is still very limited. The cytogenetic mechanisms involved in sex determination are of particular interest, as they are subject to the influence of the environment. In temperate climates aphids typically reproduce through the summer months by thelytokous parthenogenesis, producing males and sexual females only in autumn, in response to reduced photoperiod and temperature. The thelytoky is of the ameiotic or apomictic type with a single, equational maturation division (Blackman 1978). Males are XO so, in order to develop as male, an oocyte has to lose half the X chromatin of the parent female in the course of this single maturation division. According to Orlando (1974, 1983), the X chromosomes in *Megoura viciae* Buck-

ton are paired end-to-end to form a condensed, C-shaped bivalent during the growth phase of the presumptive male oocyte. They then undergo a division of the meiotic type all on their own on the maturation division spindle. First the products of pairing are separated, and then one X chromosome moves to the end of the spindle while the other divides equationally with the autosomes, to leave only one chromatid of the original X bivalent in the male egg.

Eggs are determined as male or female according to environmental conditions acting via the neuroendocrine system of the parent aphid, and the experimental induction of males by precocene in *Myzus persicae* Sulzer (Hales and Mittler 1983) indicates that juvenile hormone is involved. The critical events in sex determination, when hormonal titres are influencing X chromosome behaviour, occur during the growth phase of the oocyte. It is of particular interest to obtain a detailed description of X chromosome behaviour during this critical period, but it has hitherto been impossible to observe the chromosomes accurately in the nucleus of the growing oocyte. Here we report observations using a modified propionic acid squash technique which enables study of the prophase chromosomes in growing male and female oocytes.

Materials and methods

We chose to work with *Amphorophora tuberculata* Brown and Blackman. This aphid is ideal for such studies as it has $n = 2$, is easy to rear on excised leaves of its host plant *Geranium macrorrhizum* L., and (atypically for an aphid) readily produces males at long (16 h) as well as short (12 h) photoperiods (Brown and Blackman 1985). The position of male progeny in the reproductive sequences of apterous female aphids under two sets of conditions, 18° C with 16 h photoperiod and 16° C with 12 h photoperiod, was ascertained by caging the females individually and rearing successive batches of their progeny to adulthood. Males are produced only at a particular time in the reproductive sequence. The position of presumptive male oocytes in the ovulation sequence of each ovariole could thus be inferred from the results.

Ovaries were removed from second or third instar larvae in ice-cold saline in a 50 mm square solid watch glass. The ovarioles were cleaned of fat body and mycetome and spread out near the centre of one side of a clean 18 mm square cover slip immersed in the saline, very gently touching the germaria and youngest embryos to the cover slip

so that they adhered to the glass surface. The cover slip with attached ovarioles was touched to a paper towel to remove excess liquid and placed in ice-cold freshly mixed methanol/acetic acid (3/1). After 2 min the cover slip was transferred to fresh fixative for 30 min at 4° C, then to 75% methanol for 5 min, and then to 4 N hydrochloric acid at 28° C for 50 min. This was followed by staining with Feulgen reagent for 30–40 min at room temperature, and differentiation in distilled water. The cover slip was then examined under a binocular microscope, removing any debris and all but the germaria, prematuration follicles and very young embryos before inverting it onto a small drop of distilled water on a clean microscope slide. The slide was examined unter the low power of a compound microscope to locate follicles with oocytes at the right stage of development.

A drop of 45% propionic acid was placed at the edge of the cover slip, and selected oocytes were monitored (using a Zeiss Planapo oil-immersion objective) as the propionic acid infiltrated the preparation, swelling and clearing the cells. After observing the chromosomes in the unsquashed preparation and noting the position of the oocytes, the slide was inverted onto a filter paper and pressed to squash the ovarioles for further study and photography with high resolution phase contrast.

Results

Position of males in the reproductive sequence

Female *A. tuberculata* have ten ovarioles, five in each ovary. Ovulation starts in the thelytokous mother aphid before birth and continues through larval development, so that at the final moult to adult, each ovariole has a sequence of embryos at different stages of development, with no two embryos at exactly the same stage in either ovary. After a 2–3 day prereproductive period, progeny are born for about 40 days at the rate of about 1 per day, a rather slow rate for an aphid (Fig. 1 a, b). The sequence of female births was bimodal at both 16 h and 12 h, although the females produced at 12 h were all oviparous (and sexual) whereas those produced at 16 h were mainly viviparous (and thelytokous), except for a few oviparae late in life. A sharp dip in female births coincided with a peak in male births about 12 days after the start of larviposition.

It can be assumed, from observations of embryogenesis, that progeny are born at least approximately in the sequence that they are ovulated, from which it follows that the second egg to mature in each ovariole will usually, but not always, be male. At 16 h, each female produced 9–14 males over a longer period than at 12 h, and some of the first eggs to mature in less advanced ovarioles were male, as well as almost all the eggs maturing second in each ovariole. At 12 h only 4–9 eggs per female were determined as male and apparently these were always in the second position, but several ovarioles of each female must have matured female eggs in the second position.

In the ovaries of second and third instar larvae of *A. tuberculata* reared at 16 h and 18° C, the first embryos are at various stages of post-cleavage development and the second oocytes are at various stages of prophase through to cleavage (Fig. 2). We chose to work mainly with these instars because of the high proportion of oocytes being determined as male.
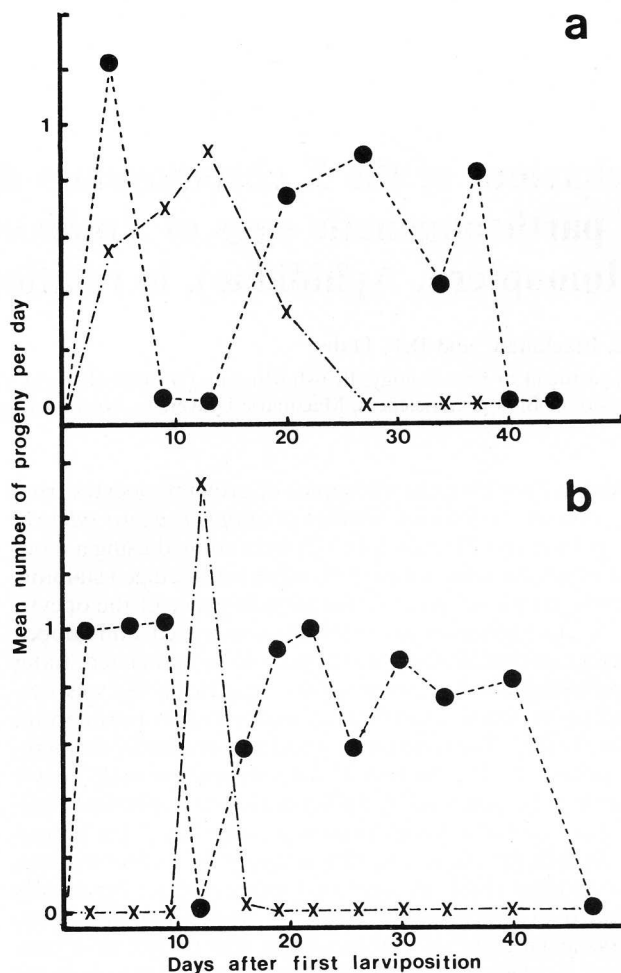

Fig. 1a, b. Progeny sequences of apterous viviparous females of *Amphorophora tuberculata* at (**a**) 16 h photoperiod, 18° C and (**b**) 12 h photoperiod, 16° C. *Crosses* are males, *solid circles* are females
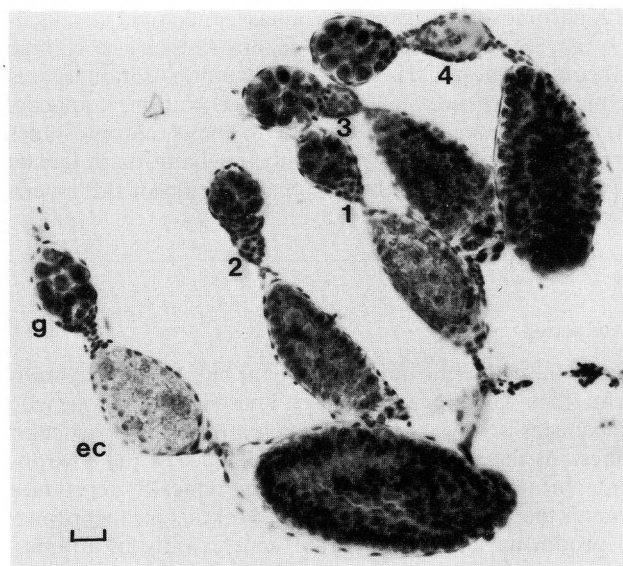

Fig. 2. One ovary of a second instar apterous viviparous female of *Amphorophora tuberculata*. The five ovarioles and the embryos within them are all at different stages of development. Prematuration oocytes in growth phase are present in four ovarioles (and numbered *1–4* in chronological order), the fifth has an egg undergoing cleavage (*ec*); *g* germarium. Feulgen staining, bright field. Bar represents 10 µm
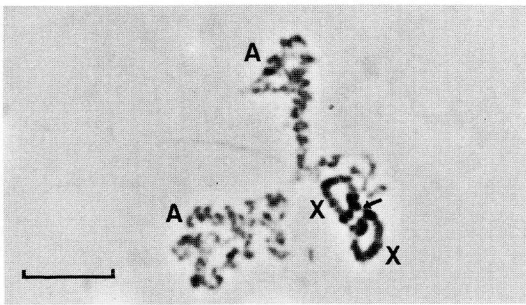
Fig. 3. Chromosomes of pre-growth phase oocyte from base of germarium of *Amphorophora tuberculata*. Feulgen staining and phase contrast. *A* autosome, *X* X chromosome. *Arrow* indicates terminal connection between X chromosomes. Bar represents 5 μm

Pre-growth phase oocytes

The ovarioles studied had all undergone at least one ovulation, and had a group of cells differentiated as presumptive oocytes at the base of the germarium (Blackman 1978). The chromosomes in all these pre-ovulation oocytes are at an early stage of prophase (Fig. 3). The chromatin of the autosomes at this stage is only partially condensed. The X chromosomes, however, are more condensed and usually

associated in a looped configuration resembling the shape of a pretzel. They appear to be joined at one end, with the remainder of each chromosome looped around so that the free ends are often located very close to the joined ends. The chromosomes of aphids, as of other Hemiptera, are holocentric.

Growth phase in female oocytes

Oocytes enter growth phase one at a time and pass out of the germarium, becoming enveloped in follicle cells. In presumptive female oocytes, the X chromosomes open out and decondense somewhat, adopting a linear end-to-end arrangement reminiscent of chromosomes with terminalised chiasmata. The terminal connection is maintained through heterochromatin (Fig. 4a–c). As the growth phase proceeds these terminal blocks also decondense, the connection is lost and the X chromosomes separate. The ends of the chromatids of each X chromosome curl away from each other appearing as two curious "horns" of unequal length (Fig. 4d, e). At the end of the growth phase the X chromosomes condense again into metaphase, when both autosomes and X chromosomes are strongly condensed (Fig. 4f). The autosomes gradually condense throughout prophase; their degree of condensation, taken together with the increasing size of the oocyte, is a useful indicator of
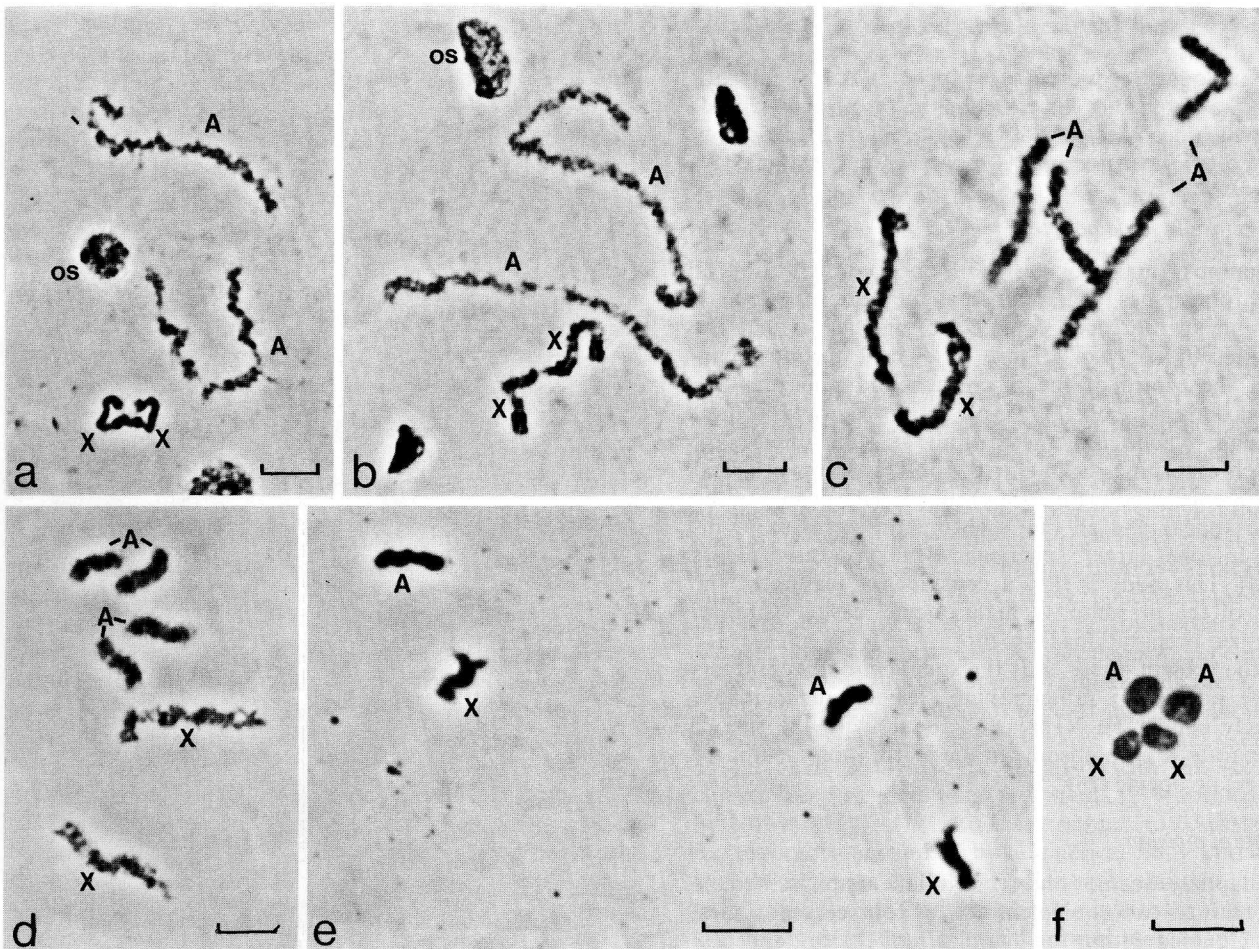

Fig. 4a–f. Stages in maturation prophase of presumptive female oocyte in *Amphorophora tuberculata*; **f** maturation metaphase. In **c** and **d** each autosome appears as two separate elements, the chromatin thread connecting them being unresolvable or broken in the preparation. Feulgen staining and phase contrast. *os* nucleus of ovariole sheath cell. Bars represent 5 μm
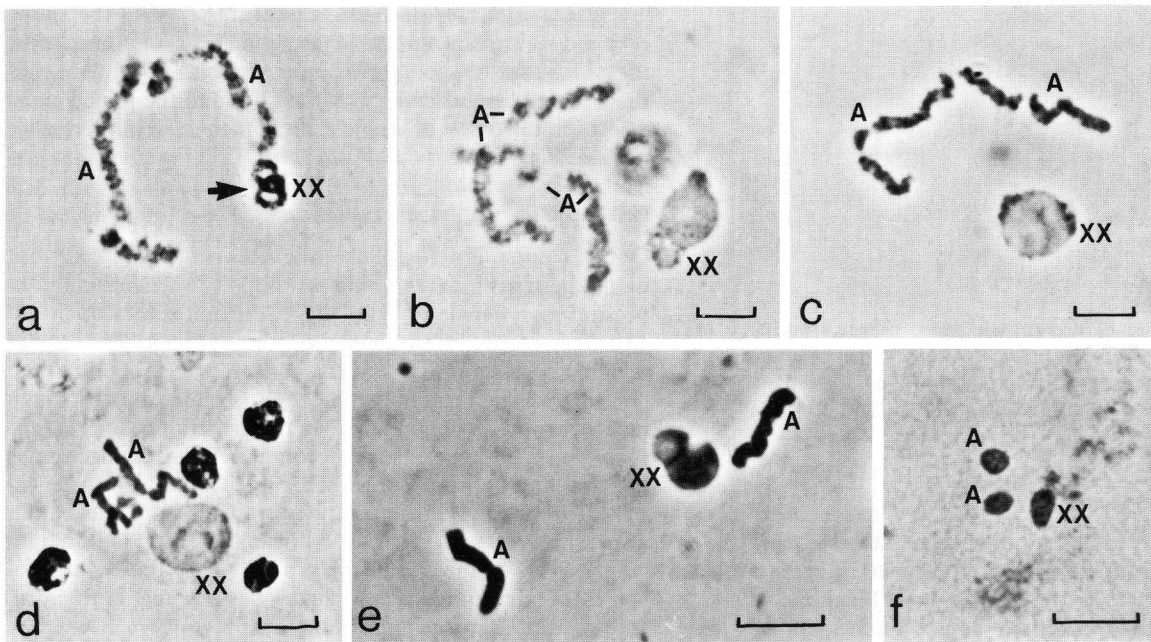
Fig. 5 a–f. Stages in maturation prophase of presumptive male oocyte in *Amphorophora tuberculata*; **f** maturation methaphase. *Arrow* indicates a developing spherical body. Dark bodies in **d** are nuclei of ovariole sheath cells. Feulgen staining and phase contrast. Bars represent 5 μm

the chronological sequence of changes in the X chromosomes. The autosomes have a constriction near their midpoints which becomes very obvious in mid-prophase, when they are often separated into two parts by squashing.

Growth phase in male oocytes

In presumptive male oocytes the looped configuration of the X chromosomes is maintained as they enter the growth phase, and all four ends come close together so that the bivalent has the shape of a figure "8". Then, starting apparently at the centre of the figure a round nucleolus-like spherical body develops (Fig. 5a), and swells rapidly until it eventually reaches a diameter of about 7 μm (Fig. 5b–d). The X chromosomes are at first visible as loops attached to the spherical body (Fig. 5b, c), but in late prophase the loops disappear and all the X chromatin seems to be involved in one large, spherical, Feulgen-positive mass (Fig. 5d). This body is thus a conspicuous feature of the nucleus of the presumptive male oocyte, easily visible in unsquashed preparations (Fig. 6).

As the growth phase ends the spherical body contracts (Fig. 5e), apparently rather rapidly, leaving at metaphase a strongly condensed X bivalent about twice the size of each separate X chromosome in the maturation metaphase of the female oocyte (Fig. 5f; cf. Fig. 4f).

Maturation division

Early anaphase was not observed. In late anaphase, female eggs contained two equivalent sets of four chromosomes, one set clumped within the cytoplasm of the egg and destined to form the female pronucleus, and the other more spaced out at the periphery of the egg, destined for enclosure in the polar body (Fig. 7a). Male eggs in contrast
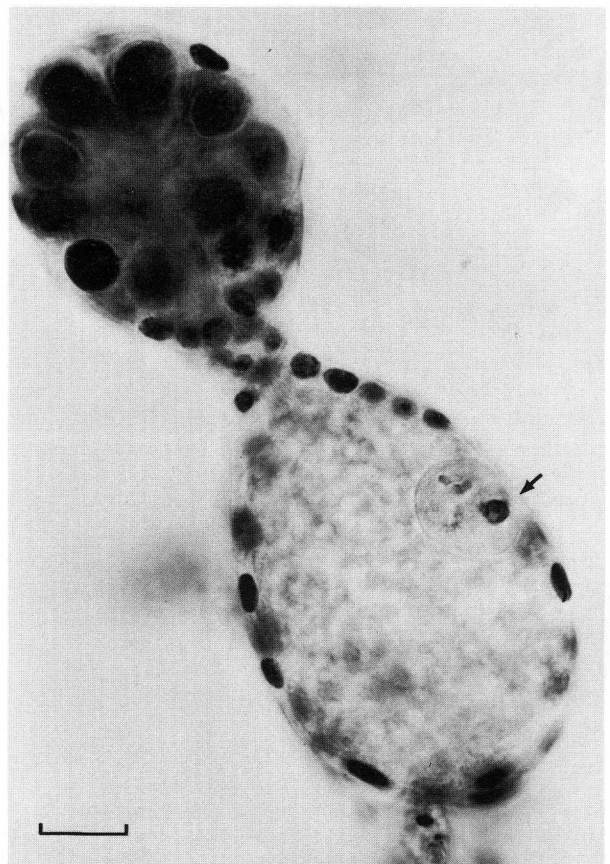

Fig. 6. Unsquashed germarium and first ovarian follicle of *Amphorophora tuberculata*, with presumptive male oocyte in late prophase of the maturation division, showing "spherical body" (*arrowed*). Feulgen staining with bright field. Bar represents 10 μm
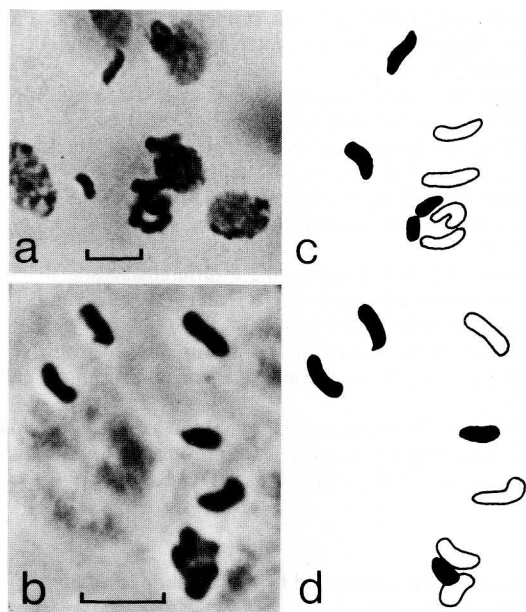
Fig. 7a–d. Late anaphase of maturation division of **a** female oocyte, and **b** male oocyte of *Amphorophora tuberculata*. Feulgen staining and phase contrast. **c, d** Interpretative drawings of same, X chromatin in *black*. The other bodies in **a** are ovariole sheath cell nuclei. Bar represents 5 μm

Fig. 8. Silver-stained female somatic cell prophase of *Amphorophora tuberculata* showing nucleolar organising regions. Bar represents 5 μm

showed one set of three chromosomes clumped within the egg (the male pronucleus) and five chromosomes at the periphery, two of these somewhat apart from the other three (Fig. 7b). We interpret the peripheral chromosomes as the three products of the equational division (one X chromosome and two autosomes), supplemented by the other, rejected, X chromosome, now divided into its two daughter elements.

Discussion

It has been shown for a number of species (Orlando and Mari 1968; Blackman 1978) that the first oocyte in an aphid ovariole develops directly from an unmodified germarial cell, and that the differentiation of the remaining germarial cells into nurse cells (trophocytes) and presumptive oocytes occurs only after the maturation of this first oocyte. In the present work we looked only at ovarioles in which the first oocyte had already matured and the germarium was already differentiated.

Prior to their growth phase no differences could be detected between oocytes destined to be male and female. All pre-growth phase oocytes at the base of the germarium have the X chromosomes looped and at least loosely associated end-to-end. This association could be a kind of "touch-and-go" pairing, as suggested by Orlando (1974), or could arise by terminalisation of a localised chiasma.

The extent of decondensation of the autosomes in these oocytes was somewhat surprising in view of observations on sectioned preparations of this stage in other species, where both autosomes and X chromosomes appeared as compact and apparently rather condensed structures (Blackman 1978). Either sectioned preparations give an inaccurate impression of the degree of condensation of the autosomes, or there are differences between *A. tuberculata*
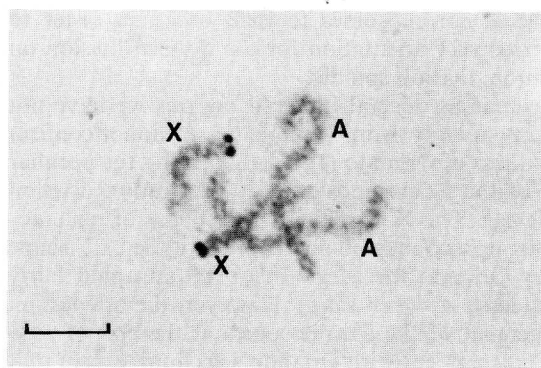
and the other species of Aphidinae studied in this respect. In *Forda marginata* Koch, an aphid in another subfamily (Pemphiginae), the chromosomes all associate together to form dense clumps in the nuclei of pre-growth phase oocytes (Blackman, unpublished observations), so it is impossible to generalise about this aspect of oocyte development.

This study confirms the findings of Orlando (1974) that the development of male and female oocytes only differs after they have begun the growth phase. The maintenance of the looped association between the ends of the X chromosomes in male oocyte development appears to be related to the development of the spherical, nucleolus-like body at the point of association (compare Figs. 4a and 5a). This continued association thus seems to be the first visible characteristic of male determination, and it may be surmised that hormone concentration in the oocyte at this time has a critical influence on whether or not the spherical body is formed.

We have detected a similar spherical body in male oocytes of *Myzus persicae* Sulzer induced both by short photoperiod and by precocene. We do not yet know its composition, nor why it should attain such a large size in male oocyte nuclei. There is reason to believe that it is partly nucleolar, as an examination of male prophase oocytes of *M. persicae* by transmission electron microscopy failed to reveal any spherical body other than the nucleolus. Silver staining shows that there is a nucleolar organising region at one end of each X chromosome in both *A. tuberculata* and *M. persicae*, where the spherical body starts to form (Fig. 8). The fact that it is Feulgen positive indicates that chromatin, perhaps as an external coating, must also be involved. The only known structures which seem comparable are those associated with XY bivalents in male meiosis I of certain other animal groups. In mammals the XY bivalent forms a sex vesicle, and at least in mice this becomes associated with nucleolar material (Oud and Reutlinger 1981). There is also some similarity with the sex "parachutes" seen in male meiosis I of many beetles (reviewed by Virkki 1984). The beetle sex parachute consists of the sex chromosomes linked by a spherical body known in at least some cases to be nucleolar, and thought in others to be a nucleolus augmented by a layer deposited on its surface. It persists into anaphase and may ensure regular segregation of the sex chromosomes.

In *A. tuberculata* the spherical body disappears at metaphase, but may serve to prolong and stabilise the attach-

ment of the X chromosomes to each other, in order to ensure their correct orientation for the double division on the maturation division spindle.

After examining several hundred oocytes we have not yet found any in early or mid-anaphase, so cannot confirm the observations of Orlando (1974) concerning the peculiar behaviour of the X chromosomes at maturation division of the male egg. The X bivalent at metaphase of male oocytes was strongly condensed and ovoid, not the "C" shape reported by Orlando for *Megoura viciae* (an aphid fairly closely related to *A. tuberculata*). However, the orientation and arrangement of the chromosomes at the end of anaphase are in agreement with Orlando's account, except that he did not observe the subsequent separation of the chromatids of the rejected X chromosome after it had moved to the periphery of the egg.

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